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Protocol for efficient plant regeneration and *Agrobacterium tumefaciens* mediated genetic transformation of pigeonpea [*Cajanus cajan* (L.) Millsp.]

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Abstract

A simple, efficient, reproducible and genotype independent high frequency plant regeneration protocol has been developed from cotyledonary node explants from 12-d-old *in vitro* raised pigeonpea seedlings cultured on shoot induction medium [Murashige and Skoog (MS) medium + 2.0 mg L^{-1} benzyladenine]. Shoot-buds originated from the cut ends of the cotyledonary node explants and multiple adventitious shoots developed from 80% of the explants. They elongated rapidly on shoot elongation medium comprising the MS medium supplemented with 0.5 mg L^{-1} gibberellic acid-A, rooted on MS medium supplemented with 0.5 mg L^{-1} indole butyric acid (IBA). The survival rate of the *in vitro* regenerated plantlets was over 70%. The cotyledonary node explants were co-cultivated with *Agrobactcrium tumefaciens* strain C-58 harboring the binary plasmid, pCAMBIA1301 [conferring β-glucuronidase (GUS) activity and resistance to hygromycin], cultured on selection medium containing hygromycin to select putatively transformed shoots and rooted. About 24 putative T0 transgenic plants have been produced and the stable expression and integration of the transgenes was confirmed by GUS assay, PCR and Southern blot hybridization with a transformation efficiency of over 45%.

Key words: *Pigeonpea, Agrobacterium tumefaciens*, genetic transformation

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is the second most important food legume of India valued as food and fodder and possesses the capacity for biological nitrogen fixation in symbiosis with *Rhizobium* sp. The constraints for enhancing its productivity include the damages caused by various fungi, bacteria, virus and insect pests. Genetic improvement of pigeonpea has been restricted due to the non-availability of better genetic resources, presence of strong sexual barriers and incompatibilities among wild relatives. Genetic engineering technology can therefore be used as an additional tool for introduction of agronomically useful traits into established cultivars.

Development of efficient plant regeneration protocols is a pre-requisite to use recombinant DNA technology to carry out genetic transformation. For successful development of transgenic plants, identification of suitable target tissue and efficient gene transfer protocols are essential [1]. Whereas, regeneration of plantlets from callus cultures is a time consuming and labor intensive task, the direct regeneration of multiple-shoots can simply be obtained from explants taken from *in vitro* germinated seedlings and uniform explant sources can be obtained at any time of the year with quick high efficiency rooting and plant regeneration [2].

Though considerable work has been done in regard to the genetic transformation of legumes, the common approach for genetic transformation was through *Agrobacterium tumefaciens* to produce transgenics such as soybean [3], chickpea [4], pea [5] and groundnut [6]. To date there are only two transformation reports in pigeonpea [7-8] however, with low transformation efficiency. Therefore, standardization of plant regeneration and transformation protocols was given major importance presently to produce transgenic pigeonpea plants on a large scale.

With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, a protocol for efficient *in vitro* plantlet regeneration from various explants has been developed and the cotyledonary node explants used as target tissue for *Agrobacterium* mediated genetic transformation to develop putatively transgenic plants. As a first step towards the development of an efficient transformation system, we transferred a marker plasmid to cotyledonary nodes of two cultivars of pigeonpea. The report describes a rapid, reliable and genotype independent protocol for *in vitro* plant regeneration and *Agrobacterium* mediated genetic transformation of pigeonpea.

Materials and methods

Plant material: Seeds of two cultivars of pigeonpea [*Cajanus cajan* (L.) Millsp.] were obtained (c.v. LRG-30 from LAM Agricultural Farm, Guntur, India and c.v. ICPL 88039 from ICRISAT, Patancheru, India). They were surface-sterilized by soaking in 70% ethanol for 5 min, washed in sterile distilled water, soaked in 0.1% (w/v) mercuric chloride containing 2 drops of Tween-20 for 10 min with intermittent shaking, rinsed thrice with sterile distilled water and soaked in distilled water for 6 h. The seed-coats of the seeds were removed aseptically and germinated on Murashige and Skoog's (MS) basal medium [9] supplemented with 3% (w/v) sucrose and 0.8% (w/v) Phyta-agar) and incubated at standard culture conditions of 16 h photoperiod at 25 ± 1°C and a light intensity of 60 μE m^{-2} S^{-1}.

Explants and culture conditions: The explants comprised of cotyledonary node (of 7-8 mm in length), primary leaf (6-7 mm in length) and shoot-tip (4 mm in length) excised from 12-day-old aseptically grown seedlings. Various Culture media comprising the MS medium supplemented with 6-benzyladenine (BA) (1.0, 2.0 and 4.0 mg L^{-1}) were experimented with to find the most suitable shoot induction medium (SIM) for induction of shoot-buds. Since gibberellic acid-A (GA_3) induced significant shoot elongation in preliminary studies, the shoots were transferred after 2 week to the GA3 containing (0.5 mg L^{-1} GA_3) shoot elongation medium (SEM). Based on certain preliminary experiments, the shoots (longer than 3 cm) were transferred to Magenta bottles containing root induction medium (RIM) *viz*: MS medium supplemented with IBA (0.5 mg L^{-1} IBA). The culture conditions employed were same as described above. Rooted plantlets transferred to pots containing a 1:1 mixture of sand and soil were acclimatized for 1 week (by covering with a plastic bag and gradually exposing the plant to open environment) prior to transfer to the glasshouse. The experiments were carried out in 3 replicates (100 explants/replicate) and statistically analyzed.

Agrobacterium strain and plasmid vector: The disarmed *Agrobacterium tumefaciens* strain C-58 harboring a binary plasmid pCAMBIA-1301 was used as vector system for transformation. The plasmid contained the reporter gene, *uidA* gene (GUS) [10] from *Escherichia coli* with an intron, driven by the cauliflower mosaic virus (CaMV) 35 S promoter and *nos* poly-A terminator sequences and the hygromycin phosphotransferase (*hpt*) gene (used as a selectable marker) under the control of CaMV 35 S promoter and (CaMV 35 S poly-A terminator (Fig. 1). Bacteria were maintained on LB [11] agar plates (1% w/v tryptone, 0.5% w/v yeast extract and 1% w/v sodium chloride, pH 7.0) with 50 μg mL^{-1} kanamycin sulfate.

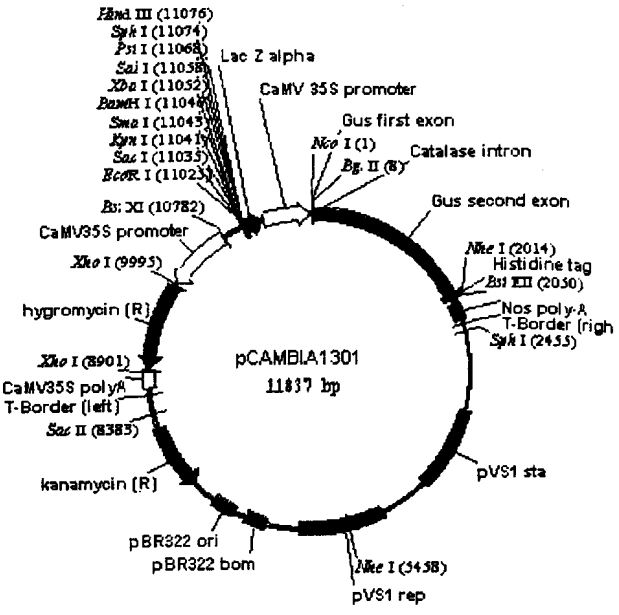

Fig. 1. Rescriction map of pCAMBIA-1301

Co-cultivation and transformation: A single bacterial colony was inoculated into 25 mL of liquid LB medium containing 50 mg L^{-1} kanamycin sulfate and incubated at 28oC on a shaker at 100 rpm for 16-18 h and used in the late log phase A600 at 0.6. The bacterial culture was centrifuged at 5000 rpm and half MS liquid medium added to the bacterial pellet to makeup a volume of 25 ml. Freshly cut explants were dipped into this suspension, blotted on sterile filter paper and transferred to SIM. Twenty explants were co-cultivated and cultured per petriplate and a total of 200 explants were used with three replicates. The co-cultivated explants were then transferred after 48 h to SIM-Cef medium comprising the SIM, supplemented with 200 mg L^{-1} cefotaxime to eliminate the bacteria.

Selection and plant regeneration: To identify the lethal concentration of hygromycin for effective selection of putatively transgenic plants, the control explants were cultured on SIM with different concentrations of hygromycin (0.5-10 mg L^{-1}). At 4 mg L^{-1} and above, the explants turned brown and did not show further growth (data not shown). Hence, 5 mg L^{-1} was used as selection pressure for the culture of co-cultivated explants. The explants cultured on SIM-Cef for 1 week were transferred to the selection medium, SIM-Sel-1 (comprising the SIM supplemented with 2 mg L^{-1} hygromycin and 200 mg L^{-1} cefotaxime) and later transferred to SIM-Sel-2 medium (after 2 weeks) comprising SIM supplemented with 5 mg L^{-1} hygromycin for 3 weeks by which time the regenerated putatively transgenic shoots would have grown considerably. The shoots were then transferred to RIM for rooting and

subsequently transferred to pots and moved to the glasshouse after acclimatization (as described previously). Control explants (not co-cultivated) were cultured simultaneously to regenerate untransformed control plants.

Histochemical GUS assay of the co-cultivated explants: Histochemical GUS assay was carried out on the co-cultivated explants with regenerated putatively transformed shoots, 4 weeks after co-cultivation by a modified method of Jefferson *et al.* [12]. The modified histochemical assay buffer consisted of 100 mM $NaPO_4$ buffer, 100 mM Na_2 EDTA,50 mM $K_4Fe(CN)_6$. 3 H_2O and 0.1% Triton X-100 (pH-7.0). 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Clontech Laboratories, Palo Alto, CA, USA) was dissolved in 50% (v/v) ethanol, stored at –20°C and added to the buffer to a final concentration of 0.5 mg mL-1 prior to the assay.

To rule out the possibility of *Agrobacterium* contamination, the putatively transformed material (2 week after co-cultivation) were cultured on antibiotic-free medium for 1 week before the analysis for GUS activity only after ascertaining that the *Agrobacterium* did not appear on the culture medium. The putatively transformed material and controls were subjected to histochemical GUS assay by scoring 1 petri plate (containing 20 explants with shoots) from each replicate (out of a total of 3 replicates). For GUS assay, the material was immersed in GUS substrate mixture immediately followed by vacuum treatment for 10 in and incubated at 37oC. Histochemical localization of GUS activity was examined under a Zeiss SV8 stereomicroscope. Chlorophyll was extracted from the material by successive incubation in 70% (v/v) ethanol for 2 h and 100% ethanol overnight to facilitate better examination. The data from the experiments were evaluated as number of GUS positive explants (explants with shoot(s) having at least one blue spot) in 20 co-cultivated explants per replicate.

Molecular analysis of putative transformants: Molecular studies were carried out to confirm the integration of foreign genes in the putatively transgenic plants. Genomic DNA was isolated from the putatively transgenic plants and untransformed control plants by a modified method of Rogers and Bendich [12].

PCR analysis: Polymerase chain reaction (PCR) analysis was carried out on the T0 putative transgenics for amplification of the coding region of *hpt* gene. One µg of RNase treated DNA was used as template for PCR amplification. Each PCR reaction was performed in 25 µl (final volume) of reaction mixture consisting of 2.5 µl 10 × PCR amplification buffer, 2 µl of template DNA, 0.5 µl 10 mM dNTPs, 0.75 µl 50 mM $MgCl_2$, 100 ng (0.5 µl) of each primer, 10.5 µl sterile distilled water, 7.5 µl enhancer (Invitrogen) and 1 unit (0.25 µl) of Platinum Taq DNA polymerase (Invitrogen). The following primers were used to amplify the 819 hp fragment of the *hpt* gene: Forward primer: 5′-CGT TAT GTT TAT CGG CAC TTTG-3′; Reverse primer: 5′-GGG GCG TCG GTT TCC ACT ATCG-3′. The samples were heated to 94°C for 4 min and then subjected to 34 cycles of 1 min at 93°C, 1 min at 58.5°C and 90 see at 72°C followed by another 5 min final extension at 72°C. The amplified products were assayed by electrophoresis on 1.5% agarose gels, visualized and photographed with ethidium bromide under ultraviolet light.

Southern blot hybridization analysis: Well-established PCR positive (*hpt*) T0 transformants were subjected to Southern blot hybridization analysis. Ten µg of genomic DNA from Putatively transformed and control plants was divested with *Xho-1*, which recognizes only two sites on either side of the *hpt* gene. The digested DNA was separated by electrophoresis through a 0.8% agarose gel and transferred onto Nylon N+ membrane (Amersham) according to manufacturer's instructions. The blot was probed with a non-radioactively labeled (Alkphos Direct Labeling and Detection system of Amersham Biosciences, Uppsala, Sweden) 819 bp PCR-amplified *hpt* gene fragment. The pCAMBIA-1301 restricted with *Xho-1* (to release the *hpt* gene) was used as positive control. The blots were exposed to X-Omat film (Kodak) for 15 m for autoradiography.

Results and discussion

Tissue culture and genetic transformation: In order to improve various traits of the plant, it is necessary to have an efficient plant regeneration system to effect any genetic manipulation through genetic engineering and transformation. In the present study, an efficient *in vitro* plant regeneration protocol from cotyledonary node explants of pigeonpea has been developed. Although response of the two genotypes was similar, more encouraging results were obtained with the cultivar, LRG-30 (Table 1). Therefore, genetic transformation was carried out on the cultivar LRG-30. The shoot induction medium (SIM) containing 2.0 mg L-1 benzyladenine was found to be most suitable and hence favoured for culture of explants after transformation (Table 1). The cotyledonary node explant was the most responsive of all the explants used and hence used for transformation (Table 1) (Fig. 2a-c). The genotype independent *in vitro* plant regeneration system developed presently from the cotyledonary node explant was rapid, reliable, reproducible and efficient. It was capable of producing plantlets independently through organogenesis and development of multiple shoots without any callus phase (Fig. 2c). The survival rate of *in vitro* regenerated plantlets was over 70%.

Table 1. The effect of different culture media on the relative frequency of plantlet regeneration via multiple shoot differentiation in two cultivars of pigeonpea

Cultivar	Explant	*Frequency of multiple shoot development on culture media containing different concentrations (mg L-1) of BA				**Frequency of rooting on MS + 0.5 mg L^{-} IBA	***Percentage of survival
		0.0	1.0	2.0	4.0		
LRG-30	Cotyledonary node	Nil	81.3 ± 0.33	95.3 ± 0.66	72.0 ± 1.73	93.0 ± 0.57	72.0 ± 0.40
	Leaf	Nil	Callus	Callus	Callus	Nil	Nil
	Shoot-tip	Nil	70.0 ± 1.15	71.3 ± 0.66	75.3 ± 0.98	70.0 ± 0.11	88.0 ± 0.70
ICPL 88039	Cotyledonary node	Nil	78.6 ± 1.02	90.0 ± 1.15	79.3 ± 0.66	89.6 ± 0.28	70.0 ± 1.52
	Leaf	Nil	Callus	Callus	Callus	Nil	Nil
	Shoot-tip	Nil	72.0 ± 1.15	74.0 ± 0.81	75.8 ± 0.55	70.6 ± 0.61	77.3 ± 0.88

*Percentage of the total cultured explants producing multiple-shoots, presented as mean ± S.E. of the three replicates; **Percentage rooting and acclimatization of the total cultured plantlets, presented as mean ± S.E. of the three replicates; ***Percentage survival of the total transplanted planticts after sixty days of growth in the glasshouse, presented as mean ± S.E. of the three replicates.

Although regeneration in pigeonpea was previously reported from cotyledonary node explants [13] and from different seedling explants [14, 15] through organogenesis and somatic embryogenesis [15, 16] the protocols were not favourable for genetic transformation, because of low regeneration frequencies and long time taken for regeneration.

GUS assay of transformants: The co-cultivated cotyledonary node explants were cultured on selection medium (containing hygromycin) to recover and regenerate transgenic plants (Fig. 2d). Successful transformation was confirmed by GUS assay, which was found to be an easy and reliable way of establishing optimal conditions for transformation. The histochemical evaluation revealed intense blue sectors in sub-epiden-nal tissue of the shoots regenerated after co-cultivation with *Agrobacterium* (Fig. 2e). Strongest GUS expression was concentrated in one area with unidirectional distribution of the blue precipitate. No blue cells were observed after histochemical staining of untransformed shoots (control). The 60% transformation efficiency presently obtained through GUS assay (Table 2) agrees with an earlier report [7]. Therefore, we conclude that transformed nodal meristem cells that produced β-glucuronidase kept their potential to undergo anticlinal divisions. Stringent hygromycin selection was applied presently to ensure the recovery of transformants with minimum escapes. While the delayed application of selective agent leads to excessive escapes [17], too early selection pressure adversely affects shoot re-generation [18]. We however found a delay period of 1 week necessary for efficient recovery of transgenics. In spite of difficulty in carrying out transplantation of transformants, a total of 24 putative T0 transformants have been established presently in the glasshouse and molecular analyses established their transgenic status.

The shoots regenerated on hygromycin selection media were rooted on RIM (Fig. 2 f-h). The putative T0 transformants transplanted to pots and moved to glasshouse flowered and produced pods normally (Fig. 2 i-j). It was extremely difficult to carry out transplantation of the transformants and the mortality of the plantlets during the hardening process was as high as 85% (data not shown). However, a total of 24 putative T0 transformants were established in the glasshouse.

Optimal conditions standardized presently for efficient transformation of the cotyledonary nodes include the use of freshly cut explants, co-cultivation duration of 48 h, a delay period of 7 d followed by culture on selection medium and application of stringent selection for three weeks before rooting.

Molecular characterization of transgenic plants: Molecular analysis of putative T0 transformants was carried out by PCR and Southern blot hybridization

Table 2. GUS assay, molecular analysis of putative transformants and transformation frequency in pigeonpea c.v. LRG-30

Expt. No.	No. of co-cultivated cytoledonaryn ode explants	No. of explants with shoots subjected to GUS assay	No. of shoots with blue spots (positive GUS assay)	No. of surviving shoots after 4 week on hygromycin selection	No. of rooted putative T0 transgenic plants	No. of putative T0 transgenic plants moved to glasshouse	No. of plants positive for PCR/total number of plants tested	No. of plants positive for Southern/ total number of plants tested	Transfor-mation frequency (%) (PCR + Southern)
1	200	20	11	111	98	10	2/10	1/3	26.66
2	200	20	13	99	82	8	3/8	2/2	68.75
3	200	20	12	98	86	6	3/6	1/2	50.00
Total transformation frequency (PCR and Southern positives)									48.47

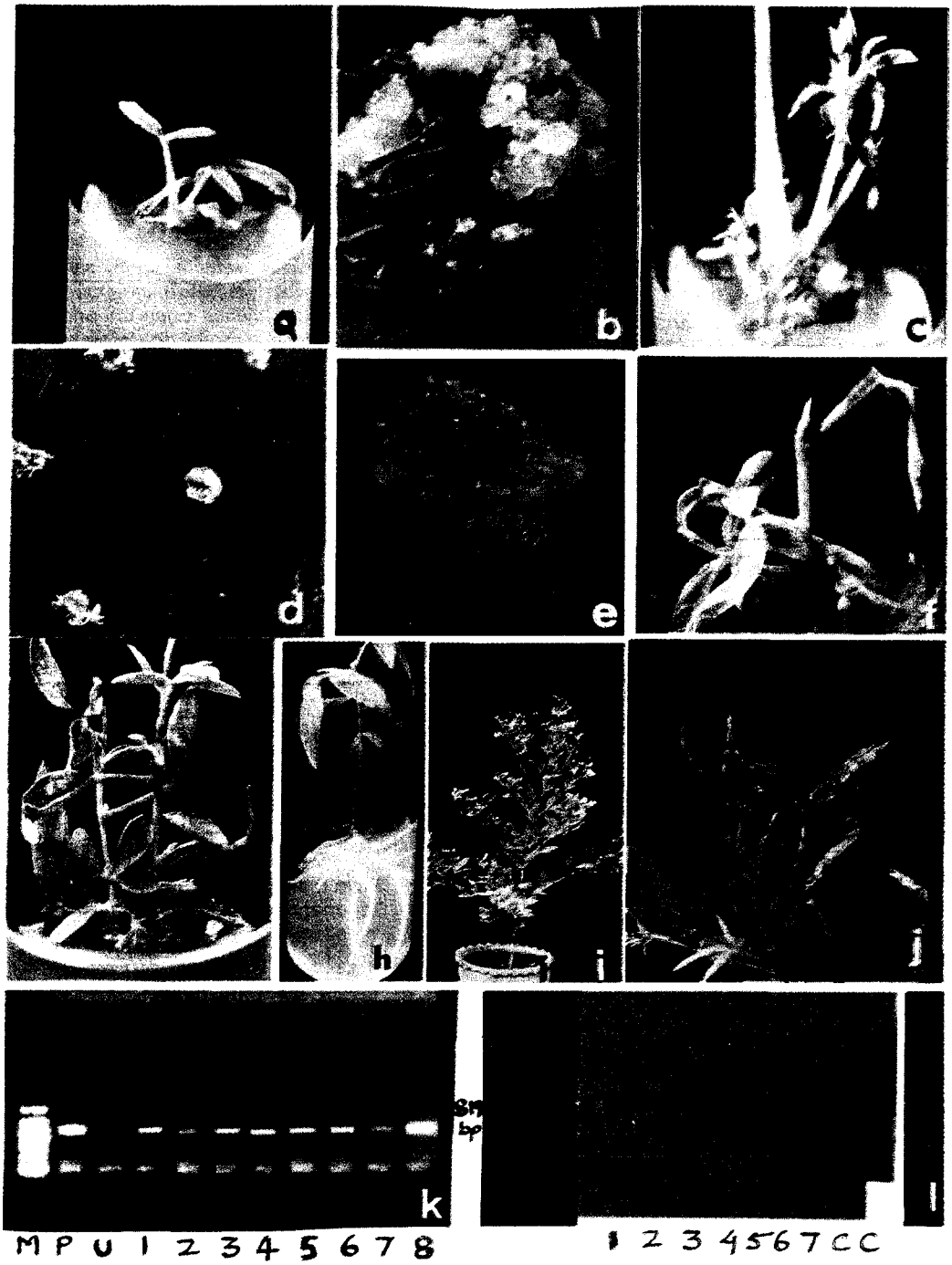

Fig. 2 a-l. Studies on *in vitro* plant regeneration, genetic transformation, and molecular analysis (PCR and Southern blotting of genomic DNA of the T0 transformants obtained via *Agrobacterium*-mediated transformation of the cotyledonary node explant using the plasmid pCAMBIA-1301) in pigeonpea: (a) Organogenesis and shoot development from shoot-tip explant of pigeonpea c.v. LRG-30; (b) Callus development from leaf explant of pigeonpea c.v. ICPL 88039; (c) Multiple shoot development from cotyledonary node explant of pigeonpea c.v. LRG-30; (d) Co-cultivation of cotyledonary node explants with *Agrobacterium tumefaciens*; (e) Results of GUS assay of transfomied explants with shoot buds; (f) Regenerated putatively (T0) transformed shoot on hygromycin selection; (g) Healthy elongated putatively (T0) transformed shoots on hygromycin selection; (h) Putatively transformed (T0) shoots with well-developed roots; (i) Flowering putative (T0) transgenic plant (P-1); (j) Putative (T0) transgenic plant (P-3) with well developed pods; (k) PCR amplification of the genomic DNA of transformants showing amplification of the 819 hp fragment of hpt gene after *Agrobacterium*-mediated gene transfer using plasmid pCAMBIA-1301. Lane 1 carries the 100 hp marker DNA (M); Lane 2 carries plasmid pCAMBIA-1301 DNA (positive control)(P); Lane 3 carries DNA from un-transformed plant (negative control)(u); Lanes 4 to 11 carry genomic DNA from 8 putative T0 plants (P-1, P-2, P-4, P-5, P-8, P-10, P-14 and P-18) transformed with plasmid PCAMBIA-1301; (l) Southern blot hybridization of hpt gene in the genomic DNA from PCR positive (T0) putative transformants. The plant genomic DNA was digested with *Xho-1* to provide a double cut within the plasmid DNA to release the *hpt* gene. The blot was probed with non-radioactive Alkphos labeled 819 hp PCR-amplified *hpt* gene fragment. Lanes 1 to 7 carry genomic DNA of 7 putative (T0) transformants (P-1, P-2, P-4, P-5, P-8, P-10, P-14); Lanes C & C carry plasmid pCAMBIA-1301 restricted with *Xho-1* to release the *hpt* gene (positive control).

(Table 2). PCR analysis was carried out for amplification of the coding region of *hpt* gene. Out of 24 transformants (T0), 8 plants were positive for the amplification of the 819 hp fragment of the *hpt* gene. (Fig. 2k).

Further, the transgene integration was confirmed in the PCR positive (*hpt*) T0 transformants through Southern hybridization analysis in which out of 7 plants, the *hpt* gene was located in 4 plants (57.1%) (Fig. 2.1). The transformation frequency analyzed through PCR and Southern hybridization was over 45%.

Our results show that by fine-tuning the conditions of transformation, even a recalcitrant crop like pigeonpea can be transformed with an optimum frequency. Although the frequency of transformation is still low compared to the model species, the protocol is repeatable and can be used to mobilize genes of agronomic importance into elite cultivars of pigeonpea.

Previous reports [7, 8] on *Agrobacterium*-mediated transformation of pigeonpea have utilized shoot apices and cotyledonary nodes to achieve direct regeneration and embryonic axes to achieve indirect regeneration through callus and reported very few plants of T0 generation with the effective frequency of transformed shoots of less than 1%. The present report is a significant improvement, with more than 45% of the transgenic plants showing positive gene integration.

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